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# Calcium-independent and cAMP-dependent Modulation of Soluble Guanylyl Cyclase Activity by G Protein-coupled Receptors in Pituitary Cells*

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**It is well established that G protein-coupled receptors stimulate nitric oxide-sensitive soluble guanylyl cyclase by increasing intracellular $Ca^{2+}$ and activating $Ca^{2+}$-dependent nitric-oxide synthases. In pituitary cells receptors that stimulated adenylyl cyclase, growth hormone-releasing hormone, corticotropin-releasing factor, and thyrotropin-releasing hormone also stimulated calcium signaling and increased cGMP levels, whereas receptors that inhibited adenylyl cyclase, endothelin-A, and dopamine-2 also inhibited spontaneous calcium transients and decreased cGMP levels. However, receptor-controlled up- and down-regulation of cyclic nucleotide accumulation was not blocked by abolition of $Ca^{2+}$ signaling, suggesting that cAMP production affects cGMP accumulation. Agonist-induced cGMP accumulation was observed in cells incubated in the presence of various phosphodiesterase and soluble guanylyl cyclase inhibitors, confirming that $G_s$-coupled receptors stimulated *de novo* cGMP production. Furthermore, cholera toxin (an activator of $G_s$), forskolin (an activator of adenylyl cyclase), and 8-Br-cAMP (a permeable cAMP analog) mimicked the stimulatory action of $G_s$-coupled receptors on cGMP production. Basal, agonist-, cholera toxin-, and forskolin-stimulated cGMP production, but not cAMP production, was significantly reduced in cells treated with H89, a protein kinase A inhibitor. These results indicate that coupling seven plasma membrane-domain receptors to an adenylyl cyclase signaling pathway provides an additional calcium-independent and cAMP-dependent mechanism for modulating soluble guanylyl cyclase activity in pituitary cells.**

Soluble guanylyl cyclases (sGC)[1] catalyze the formation of cGMP in response to a wide variety of agents including hormones and neurotransmitters acting through G protein-coupled receptors (GPCRs). Stimulation of sGC is especially well established for GPCRs that signal through phospholipase C- and adenylyl cyclase (AC)-dependent pathways. It is generally accepted that calcium mediates the coupling of these receptors to sGC (1). Activation of the phospholipase C signaling pathway leads to inositol trisphosphate-induced $Ca^{2+}$ mobilization accompanied by facilitation of voltage-sensitive and/or -insensitive $Ca^{2+}$ influx (2), whereas activation of the AC signaling pathway usually facilitates $Ca^{2+}$ influx without affecting $Ca^{2+}$ mobilization (3). Both cAMP and protein kinase A stimulate voltage-gated $Ca^{2+}$ influx, the former through activation of cyclic nucleotide-gated channels (4) and the latter by activating nonselective cationic channels (5, 6). The rise in intracellular calcium concentration ($[Ca^{2+}]_i$) induced by these receptors is sufficient to stimulate two nitric-oxide (NO) synthase (NOS) enzymes, endothelial (eNOS) and neuronal (nNOS) (7). Because sGC is activated by NO, either as an intracellular or plasma membrane permeable agonist (8), calcium-dependent NO production should result in stimulation of sGC.

Several lines of evidence support the operation of the NOS/sGC signaling system in the anterior pituitary. In normal and immortalized pituitary cells, calcium-sensitive nNOS and eNOS were detected (9–15). The activity of these enzymes was confirmed by measurements of NO, $NO_2$, and $NO_3$ under different experimental paradigms (16, 17). sGC is also expressed in pituitary tissue and dispersed cells, enriched lactotrophs and somatotrophs, and $GH_3$-immortalized cells. This enzyme is exclusively responsible for cGMP production in unstimulated cells (18). Consistent with the $Ca^{2+}$-calmodulin sensitivity of nNOS (19), agonists that increase $[Ca^{2+}]_i$, including the $Ca^{2+}$-mobilizing thyrotropin-releasing hormone (TRH) and $Ca^{2+}$ influx-dependent growth hormone-releasing hormone (GHRH), were found to modulate NO levels and hormone secretion (10, 17, 20–23). Furthermore, basal sGC activity is partially dependent on spontaneous voltage-gated calcium influx (18). These findings support a generally accepted view about the relevance of calcium in activation of the NOS/sGC signaling pathway by GPCRs. Recent studies have also indicated that high $[Ca^{2+}]_i$ inhibits cGMP accumulation, an action presumably mediated by direct inhibitory effects of $[Ca^{2+}]_i$ on sGC activity (24, 25).

Here we addressed the hypothesis that cAMP, in addition to $[Ca^{2+}]_i$, mediates the action of GPCRs on sGC-controlled cGMP production. Experiments were done in cultured pituitary cells and immortalized $GH_3$ cells. We focused investigations on two receptors signaling through a $G_s$ pathway, GHRH and corticotropin-releasing factor (CRF) receptors; two receptors signaling through a $G_q/G_{11}$ pathway, TRH receptor (that also couples to a $G_s$ signaling pathway) (26) and the endothelin (ET)-A receptor that couples to a $G_i/G_o$ signaling pathway (27, 28); and the

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[1] The abbreviations used are: sGC, soluble guanylyl cyclase; GPCRs, G protein-coupled receptors; GHRH, growth hormone-releasing hormone; CRF, corticotropin-releasing hormone; TRH, thyrotropin-releasing hormone; ET, endothelin; NOS, nitric-oxide synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; AC, adenylyl cyclase; PDEs, phosphodiesterases; IBMX, 3-isobutyl-1-methylxanthine; CTX, cholera toxin; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; NS 2028, 4H-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one; 8-Br-cAMP, 8-bromo-cAMP.

dopamine $D_2$ receptor that signals through a $G_i/G_o$ pathway without activating phospholipase C (29). GHRH receptors are expressed in somatotrophs and immortalized $GH_3$ pituitary cells (30). CRF receptors are expressed in corticotrophs (31). TRH receptors are expressed in thyrotrophs, lactotrophs, and $GH_3$-immortalized cells (32). $D_2$ receptors are expressed in lactotrophs and somatotrophs (29), whereas $ET_A$ receptors are expressed in all secretory cell types (33–35).

## MATERIALS AND METHODS

*Cell Cultures and Treatments*—Experiments were performed on anterior pituitary cells from normal female Sprague-Dawley rats obtained from Taconic Farms (Germantown, NY) and immortalized $GH_3$ cells. Pituitary cells were dispersed as described previously (36). Cells were cultured in medium 199 containing Earle's salts, sodium bicarbonate, 10% horse serum, and antibiotics. $GH_3$ cells were cultured in F12K nutrient mixture containing 1.5 g/liter $NaHCO_3$, 2.5% fetal bovine serum, and 15% horse serum. Cells were stimulated by GHRH, CRF, TRH (from Bachem, Torrance, CA), forskolin, and 8-Br-cAMP (from RBI, Natick, MA). Phosphodiesterases (PDEs) were inhibited by 3-isobutyl-1-methylxanthine (IBMX), dipyridamole, and zaprinast from Sigma-RBI. Two compounds, 4H-8-bromo-1,2,4-oxadiazolo(3,4-d)benz-(b)(1,4)oxazin-1-one (NS 2028) from Sigma-RBI and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) from Sigma were used to inhibit sGC.

*cGMP and cAMP Measurements*—Cells ($1 \times 10^6$ per well) were plated in 24-well plates in serum-containing M199 and incubated overnight at 37 °C under 5% $CO_2$/air and saturated humidity. Experiments were done in cells 16 h after dispersion. Prior to the experiments, medium was removed, and cells were washed with serum-free M199 and stimulated at 37 °C under 5% $CO_2$/air and saturated humidity. Cells were stimulated with GHRH, CRF, and TRH in serum-free medium and in the absence or presence of PDE inhibitors. At the end of the incubation period cells were dialyzed in an IBMX-containing medium, and cyclic nucleotide concentrations were determined by radioimmunoassay in medium and in dialyzed cells as previously described (24) using specific antisera provided by Albert Baukal, (NICHD, National Institutes of Health, Bethesda, MD).

*Measurements of Intracellular Calcium Ion Concentration*—For $[Ca^{2+}]_i$ measurements, cells were incubated in Krebs-Ringer buffer supplemented with 2 $\mu$M fura-2/AM (Molecular Probes, Eugene OR) at 37 °C for 60 min. Coverslips with cells were washed with this buffer and mounted on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to the Attofluor Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). Cells were examined under a ×40 oil immersion objective during exposure to alternating 340 and 380 nm light beams, and the intensity of light emission at 520 nm was measured. The ratio of light intensities, $F_{340}/F_{380}$, which reflects changes in $Ca^{2+}$ concentration, was followed in several single cells simultaneously.

*Calculations*—cAMP and cGMP data are shown as total (cell content + medium) nucleotide levels. The results shown are means ± S.E. from sextuplicate determination in one of at least three similar experiments. Asterisks indicate a significant difference among means, estimated by Student's *t* test.

## RESULTS

*GPCRs Stimulate sGC Activity*—In a mixed population of pituitary cells bathed in medium containing no PDE inhibitors, basal cGMP was about 1–2 pmol/well (Fig. 1*A*, *right panel*). Addition of 1 mM IBMX, a nonselective inhibitor of PDEs, increased cGMP levels to about 10 pmol/well (Fig. 1*B*, *right panel*) suggesting that PDEs participate in the control of basal cGMP levels in pituitary cells. As shown in Fig. 1*C*, *right panel*, no further increase in cGMP levels was observed in cells bathed in medium containing 1 mM IBMX together with two cGMP-specific PDE inhibitors, dipyridamole (50 $\mu$M) and zaprinast (50 $\mu$M). Basal cAMP levels also increased in cells bathed in 1 mM IBMX-containing medium (Fig. 1, *A versus B*, *left panels*), whereas the addition of all three PDE inhibitors did not further elevate cAMP levels (Fig. 1*C*, *left panel*). These results suggest that 1 mM IBMX is sufficient to silence PDEs in anterior pituitary cells.

GHRH, CRF, and TRH induced significant elevations in cGMP levels comparable to basal levels. As shown in Fig. 1, *right panels*, all three agonists stimulated cGMP accumulation when PDEs were not silenced (*A*), but also in cells bathed in IBMX-containing medium (*B*), and medium containing a mixture of three inhibitors (*C*). Agonist-induced cGMP accumulation was also observed in cells treated with 50 $\mu$M zaprinast and 50 $\mu$M dipyridamole alone (Table I). GHRH-induced cGMP accumulation was observed in cells bathed in medium containing 10 $\mu$M vinpocetine, a specific PDE1 inhibitor (basal, 3.44 ± 0.04; 1 nM GHRH-treated, 5.04 ± 0.235 pmol/well; $p < 0.05$). GHRH, CRF, and TRH also induced a significant and PDE-independent increase in cAMP accumulation (Fig. 1, *left panels* and Table I). These data indicate that an agonist-induced rise in cGMP levels does not result from modulation of PDE activity but represents *de novo* cGMP production.

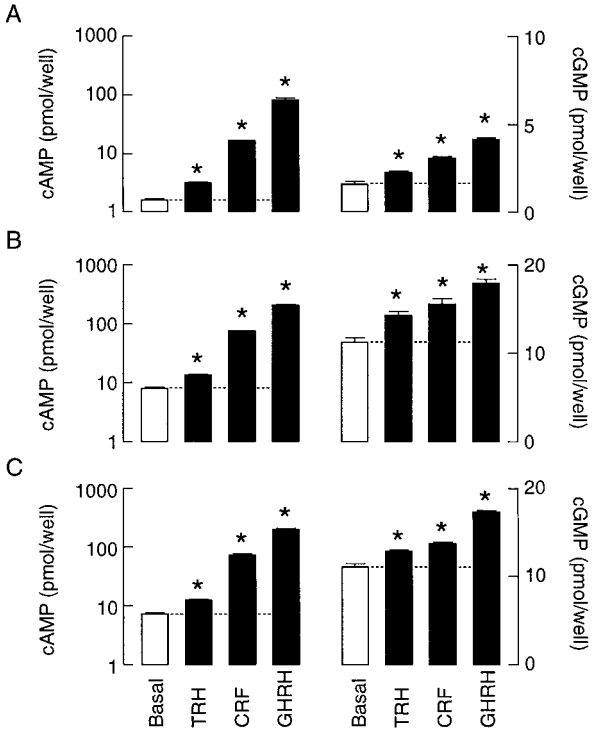


FIG. 1. **Agonist-induced cyclic nucleotide production in pituitary cells.** Effects of 100 nM GHRH, CRF, and TRH on cAMP (*left panels*) and cGMP (*right panels*) accumulation during a 60-min incubation in medium containing no PDE inhibitors (*A*), 1 mM IBMX (*B*), and 1 mM IBMX, 50 $\mu$M dipyridamole, and 50 $\mu$M zaprinast (*C*). In this and the following figures, data shown are means ± S.E. from sextuplicate incubations in one from at least three similar experiments. cAMP and cGMP were determined from the same samples. *Asterisks* indicate significant differences between untreated and agonist-treated cells, $p < 0.05$.

To identify which guanylyl cyclase, particulate or soluble, is responsible for agonist-induced cGMP production, cells were incubated in the presence of ODQ and NS 2028, two inhibitors of sGC (37, 38). As shown in Fig. 2*A*, *left panel*, ODQ significantly inhibited basal and GHRH-induced cGMP production in two doses, 0.1 $\mu$M and 1 $\mu$M, in cells bathed in medium without PDE inhibitors. NS 2028 also inhibited basal and GHRH-induced cGMP accumulation in a concentration-dependent manner with an estimated $EC_{50}$ of about 100 nM when cells were bathed in 1 mM IBMX-containing medium (Fig. 2*B*, *left panel*). None of these inhibitors affected basal and GHRH-induced cAMP production (right panels). NS 2028 also inhibited CRF and TRH-induced cGMP production when added in 1 $\mu$M concentration (not shown). These results indicate that basal and agonist-induced cGMP production in pituitary cells is mediated by sGC.

TABLE I

*Effects of dipyridamole (50 μM) and zaprinast (50 μM) on agonist (100 nM)-induced cyclic nucleotide accumulation in anterior pituitary cells during a 60-min incubation*

| Agonists | Dipyridamole | | Zaprinast | |
|---|---|---|---|---|
| | cAMP | cGMP | cAMP | cGMP |
| | *pmol/well* | | | |
| Basal | 3.28 ± 0.16 | 3.62 ± 0.38 | 0.98 ± 0.17 | 3.51 ± 0.49 |
| TRH | 8.28 ± 0.84[a] | 4.46 ± 0.13[a] | 2.18 ± 0.29[a] | 5.74 ± 0.05[a] |
| CRF | 24.59 ± 3.96[a] | 6.23 ± 0.34[a] | 16.37 ± 2.84[a] | 6.73 ± 0.31[a] |
| GHRH | 157 ± 6.49[a] | 8.36 ± 0.22[a] | 97.89 ± 4.29[a] | 8.63 ± 0.41[a] |

[a] $p < 0.05$ vs. basal.

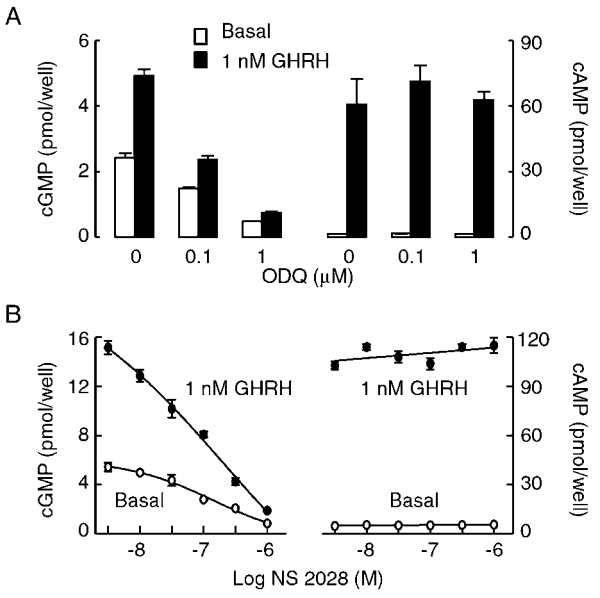


FIG. 2. **Effects of sGC inhibitors on GHRH-induced cyclic nucleotide production in pituitary cells.** *A*, concentration-dependent effects of ODQ on cGMP (*left panel*) and cAMP (*right panel*) production in unstimulated (*Basal*) and GHRH-stimulated cells bathed in medium without PDE inhibition. *B*, concentration-dependent effects of NS2028 on cGMP (*left panel*) and cAMP (*right panel*) production in cells stimulated with GHRH in 1 mM IBMX-containing medium.

*Calcium Independence of Agonist-induced cGMP Production*—GHRH and CRF increased $[Ca^{2+}]_i$ in cells bathed in 2 mM $Ca^{2+}$-containing medium (Fig. 3, *A* and *B*, *left traces*) but not in 10 μM $Ca^{2+}$-containing medium (*right traces*), indicating that $[Ca^{2+}]_i$ transients induced by these agonists were dependent on $Ca^{2+}$ influx. TRH-induced $Ca^{2+}$ response (Fig. 3*C*, *left trace*) was not abolished by depletion of extracellular $Ca^{2+}$ (not shown). However, when cells were bathed in $Ca^{2+}$-deficient medium in the presence of 1 μM thapsigargin for 30 min prior to addition of TRH, no response in $[Ca^{2+}]_i$ was observed (*right trace*) indicating that the $Ca^{2+}$-signaling action of this agonist was abolished when the intracellular $Ca^{2+}$ pool was depleted.

In cells bathed in $Ca^{2+}$-deficient medium, GHRH and CRF induced a significant increase in cGMP accumulation (Fig. 3, *D* and *E*, *right panels*). TRH was also able to stimulate cGMP production when bathed in $Ca^{2+}$-deficient and thapsigargin-containing medium (Fig. 3*F*, right panel). Agonist-induced cAMP accumulation was also not blocked by abolition of the $Ca^{2+}$-signaling function of these receptors (Fig. 3, *D–F*, *left panel*). These results indicate that all three GPCRs can stimulate cGMP production and cAMP production in a $[Ca^{2+}]_i$-independent manner.

*Correlation between cAMP and cGMP Production*—When stimulated with 100 nM agonist concentrations, the amplitudes of cGMP responses were highest in GHRH-stimulated cells followed by CRF and TRH. The same order was observed in cells bathed in $Ca^{2+}$-containing medium with and without PDE inhibitors (Fig. 1) and in cells bathed in $Ca^{2+}$-deficient medium (Fig. 3). In all experimental conditions cGMP production paralleled cAMP production raising the possibility that cAMP, in addition to $[Ca^{2+}]_i$, mediates the action of these receptors on cGMP production.

To further compare cAMP and cGMP production, cells bathed in 1 mM IBMX-containing medium were stimulated with increasing concentrations of GHRH, CRF, and TRH, and both nucleotides were measured from the same samples. Fig. 4*A* illustrates the concentration-dependent effects of these agonists on cAMP production (*left panel*) and cGMP production (*right panel*). In all concentrations tested the $Ca^{2+}$-mobilizing TRH was less potent stimulating cGMP production and cAMP production compared with CRF and GHRH. Correlation analysis of these data combined revealed the existence of a significant linear relationship between cAMP and cGMP production (Fig. 4*B*).

Further experiments were done with $GH_3$-immortalized cells expressing both GHRH and TRH receptors. As in pituitary cultured cells, GHRH and TRH stimulated cAMP and cGMP production in a concentration-dependent manner (Fig. 4*C*). There was also a parallel in cAMP and cGMP accumulation, as illustrated by linear regression in Fig. 4*D*, derived from these two experiments. In contrast to primary culture, in $GH_3$ cells GHRH- and TRH-induced cAMP and cGMP responses were almost comparable, suggesting that the cross-coupling of TRH receptors to $G_s$ is more effective in these cells than in pituitary cells.

*Dependence of Receptor-induced cGMP Production on $G_s$ Coupling*—Dissociation between $Ca^{2+}$ signaling and cGMP production and parallelism between cAMP and cGMP production suggest that coupling of GHRH, CRF, and TRH receptors to the $G_s$ signaling pathway accounts for their actions on cGMP production. To test this hypothesis more directly, cells were treated with 10 ng/ml CTX, an activator of $G_s$, for variable times. During a 2-h incubation in the presence of 1 mM IBMX this treatment significantly increased cAMP production (Fig. 5*A*, *left panel*) as well as cGMP production (Fig. 5*A*, *right panel*). As in agonist-stimulated cells (Fig. 4), there was a linear relationship between cAMP and cGMP levels in untreated (Fig. 5*B*, *left panel*) and CTX-treated cells (*right panel*). In cells treated with CTX for 12 h and 24 h without IBMX, then washed, and incubated for an additional 60 min in the presence of 1 mM IBMX, cAMP production was several fold higher than in controls (Fig. 5*C*, *left panel*). In these cells cGMP production was also significantly elevated (*right panel*). The addition of 1 μM forskolin, an AC activator (Fig. 5*D*, *left panel*), and 8-Br-cAMP, a permeable cAMP analog, also induced the time-dependent rise in cGMP production in cells bathed in 1 mM IBMX-contained medium (Fig. 5*D*, *right panel*). Thus, the non-receptor-mediated activation of the $G_s$-AC signaling pathway also leads to stimulation of sGC activity.

To test the specificity of AC-dependent signaling in GPCR-controlled cGMP production, in further experiments we stimulated cells with ET-1, an agonist for $ET_A$ receptors and apo-

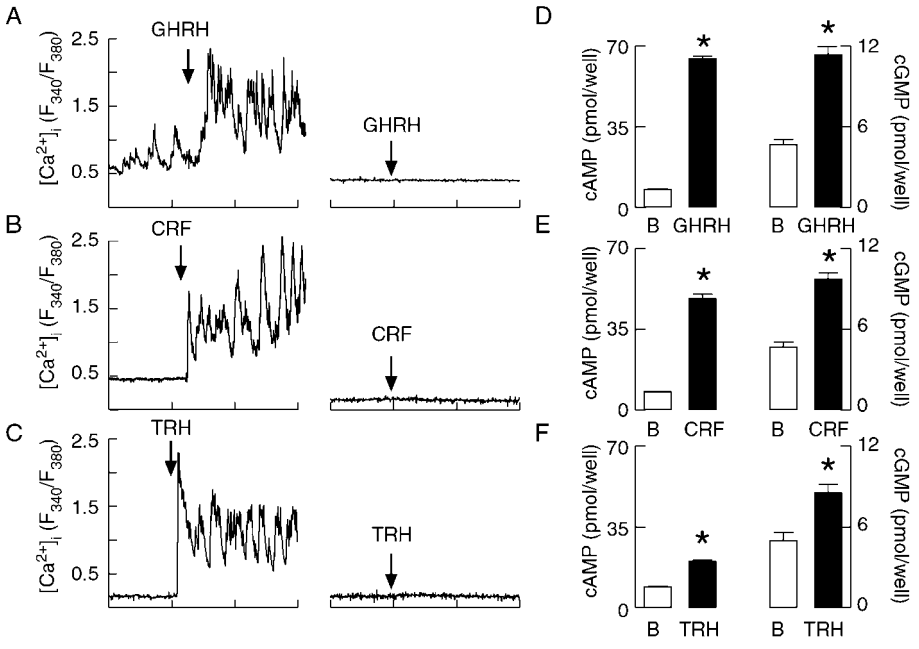


FIG. 3. **Independence of agonist-induced cyclic nucleotide production of calcium signaling in pituitary cells.** *A–C*, *left traces*, agonist-induced rise in $[Ca^{2+}]_i$ in somatotrophs (*A*), corticotrophs (*B*), and lactotrophs (*C*) bathed in medium containing 2 mM $Ca^{2+}$. *Right traces*, the lack of effects of agonists on $[Ca^{2+}]_i$ in cells bathed in $Ca^{2+}$-deficient medium (*A* and *B*) and in cells treated for 30 min with 1 $\mu$M thapsigargin in $Ca^{2+}$-deficient medium (*C*). *D–F*, agonist-induced cyclic nucleotide production in cells bathed in $Ca^{2+}$-deficient medium (*D* and *E*) and $Ca^{2+}$-deficient medium in the presence of thapsigargin (*F*). The estimated $Ca^{2+}$ concentration in $Ca^{2+}$-deficient medium was about 10 $\mu$M. In these and experiments shown in Figs. 4–7, incubation was done in medium containing 1 mM IBMX. *B*, basal cyclic nucleotide production.

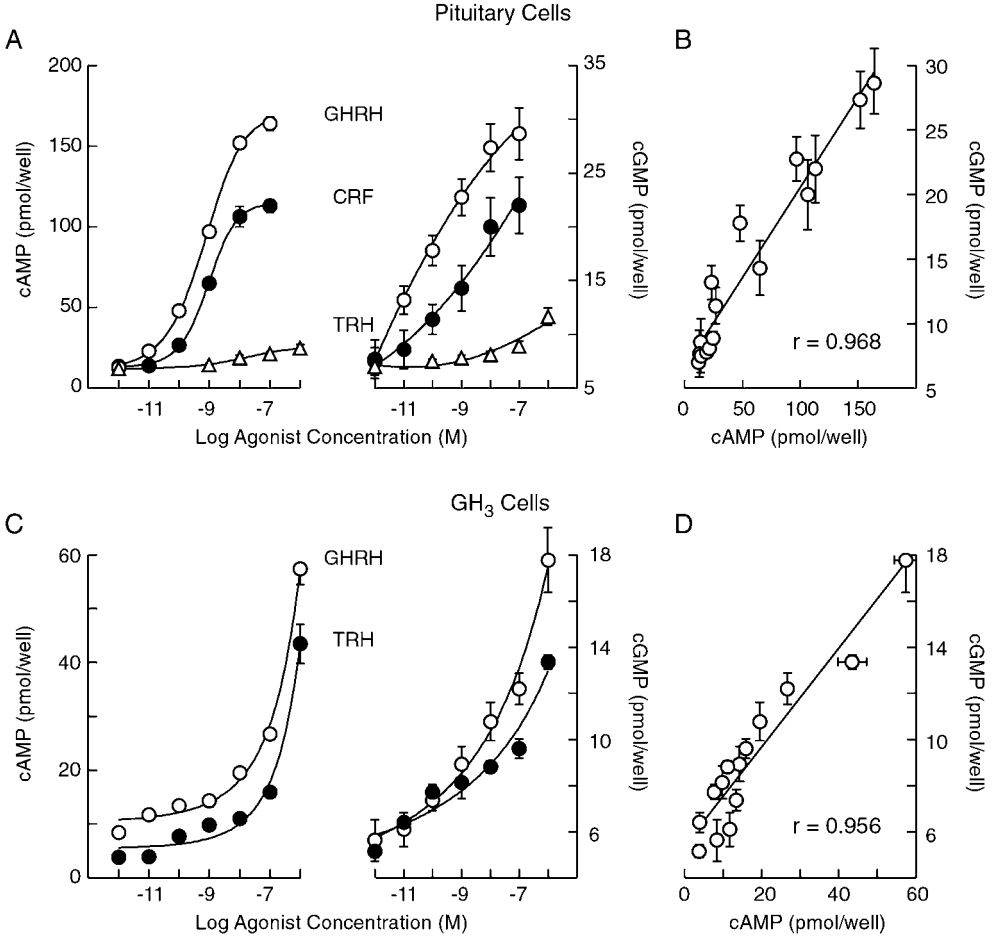


FIG. 4. **Parallelism in cAMP and cGMP production in agonist-stimulated pituitary cells.** *A*, concentration-dependent effects of GHRH (*empty circles*), CRF (*filled circles*), and TRH (*triangles*) on cyclic nucleotide production in pituitary cells. *B*, correlation between cAMP and cGMP levels. Derived from experiments shown in *panel A*. *C*, concentration-dependent effects of GHRH (*empty circles*) and TRH (*filled circles*) on cyclic nucleotide production in $GH_3$-immortalized pituitary cells. *D*, correlation between cAMP and cGMP levels. Derived from experiments shown in *panel C*. *r*, Pearson's coefficient of correlation.

morphine, a specific agonist for $D_2$ receptors. Both agonists abolished spontaneous $[Ca^{2+}]_i$ transients in pituitary cells (Fig. 6, *A* and *B*). ET-1, but not apomorphine, also stimulated $Ca^{2+}$ mobilization resulting in a rapid and transient $[Ca^{2+}]_i$ response. In accord with literature data (27, 39), ET-1 and apomorphine inhibited cAMP production in a concentration-dependent manner (Fig. 6*C*), and this inhibition was accompanied by a decrease in cGMP production (Fig. 6*D*). Con-

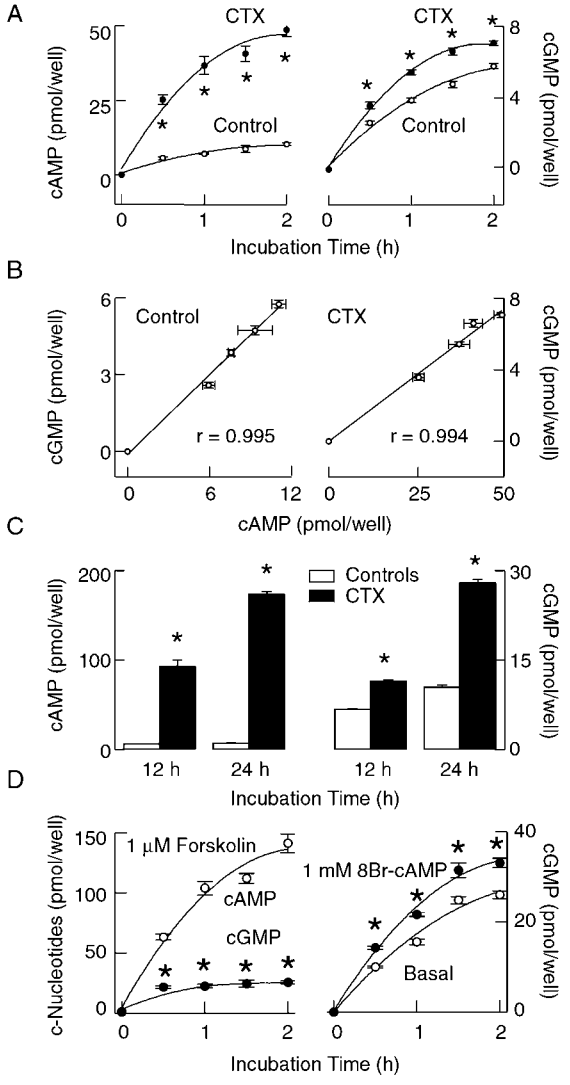


FIG. 5. **Dependence of cGMP production on $G_s$ signaling pathway in pituitary cells.** *A*, short-term effects of CTX on cAMP (*left panel*) and cGMP (*right panel*) production in pituitary cells. *B*, correlation between cAMP and cGMP levels in untreated (*left panel*) and CTX-treated (*right panel*) cells. Derived from experiments shown in *panel A*. *C*, long term effects of CTX on cAMP (*left panel*) and cGMP (*right panel*) levels. Cells were treated with 10 ng/ml CTX or solvent for 12 or 24 h, washed, and incubated for an additional 60 min. *D*, dependence of cGMP production on cAMP levels in pituitary cells. *Left panel*, time course of forskolin effects on cAMP and cGMP production. Basal cAMP and cGMP levels were subtracted. *Right panel*, time course of 8-Br-cAMP effects on cGMP production. *Asterisks* indicate significant differences between the pairs, $p < 0.05$.

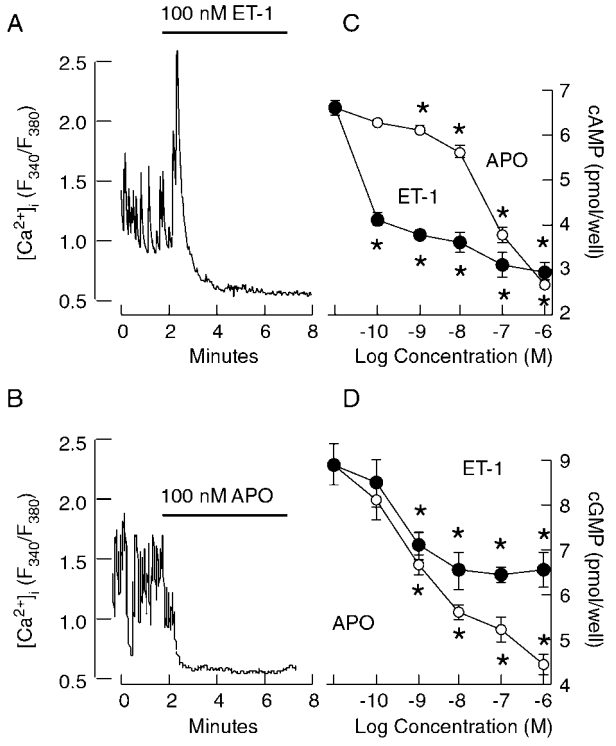


FIG. 6. **Characterization of ET-1 and apomorphine actions on calcium signaling and cyclic nucleotide production in pituitary cells.** *Left panels*, inhibition of spontaneous $[Ca^{2+}]_i$ transients by ET-1 (*A*) and apomorphine (*APO*) (*B*). *Right panels*, inhibition of cAMP (*C*) and cGMP (*D*) production by ET-1 and apomorphine. *Asterisks* indicate significant differences between the pairs, $p < 0.05$.

sistent with the role of $Ca^{2+}$ mobilization in activation of NOS/sGC (7), inhibition of cGMP production was less prominent in ET-1 than apomorphine-stimulated cells.

To test the hypothesis that protein kinase A mediates the action of cAMP on stimulation of sGC activity, cells were treated with 1 $\mu$M H89, a concentration that predominantly inhibits protein kinase A. Addition of H89 for 15 min prior to stimulation with agonists resulted in a significant reduction of receptor-induced cGMP production (Fig. 7*A*). Forskolin and CTX-induced cGMP production was also reduced in the presence of 1 $\mu$M H89 (Fig. 7*B*). On the other hand, cAMP levels measured in the same samples were not affected by H89 (Table II).

## DISCUSSION

It is generally accepted that GPCRs stimulate NO-sensitive sGC by increasing $[Ca^{2+}]_i$ and activating $Ca^{2+}$-dependent nNOS and/or eNOS (1, 18). In accordance with this hypothesis, earlier studies have indicated that sGC is expressed in pituitary cells as well as nNOS and eNOS, and that spontaneous electrical activity and calcium signaling provide an effective mechanism for activation of NOS and subsequent stimulation of sGC (18, 24). Here we extended these investigations by studying the actions of several GPCRs on cGMP production. The focus in investigations was on receptors that stimulate and inhibit spontaneous, voltage-gated calcium influx-dependent $[Ca^{2+}]_i$ transients, as well as on receptors that facilitate calcium release from intracellular $Ca^{2+}$ pools.

In cultured pituitary cells, $ET_A$ and $D_2$ receptors inhibited spontaneous $[Ca^{2+}]_i$ transients and decreased cGMP production, whereas GHRH, CRF, and TRH elevated $[Ca^{2+}]_i$ and stimulated cGMP production. Both $ET_A$ and $D_2$ receptors signal through $G_{i/o}$ pathways leading to inhibition of AC and stimulation of inward rectifier potassium current that hyperpolarizes cells, terminates spontaneous firing of action potentials, and abolishes voltage-gated $Ca^{2+}$ influx (27, 39). On the other hand, GHRH and CRF receptors stimulate AC activity through $G_s$ (30, 31). Somatotrophs respond to GHRH with a robust increase in cAMP production and stimulation of nonselective cationic channels, presumably through protein kinase A, depolarization of cells, and facilitation of voltage-gated $Ca^{2+}$ influx (5, 6, 40–42). CRF receptors also increase excitability of corticotrophs and facilitate voltage-gated calcium influx (43, 44). Finally, TRH receptor is a member of the $G_{q/11}$-coupled calcium-mobilizing receptors whose activation leads to inositol trisphosphate-induced $Ca^{2+}$ release followed by facilitated calcium influx (32). This receptor is also cross-coupled to the $G_s$-AC signaling pathway (26), and protein kinases A and C pathways may play an important role in controlling the sustained voltage-gated $Ca^{2+}$ influx by inhibiting spontaneously active inward-rectified potassium channels (3). Thus, $Ca^{2+}$ sig-

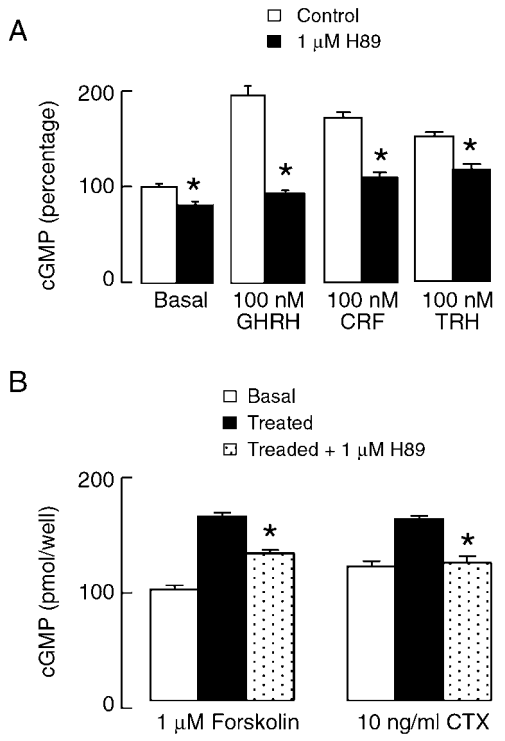


FIG. 7. **Dependence of cGMP production on protein kinase A activation.** *A*, effects of H89 on basal and agonist-induced cGMP production. *B*, effects of H89 on forskolin and CTX-induced cGMP production. Asterisks indicate significant differences between stimulated cells in the presence and absence of H89.

TABLE II
*cAMP levels in anterior pituitary cells treated with agonists (100 nM) for 60 min in 1 mM IBMX-containing medium in the absence and presence of 1 μM H89*

| Agonists (100 nM) | −H89 | +H89 |
|---|---|---|
| | *pmol/well* | |
| GHRH | 65.45 ± 1.32 | 63.83 ± 2.81 |
| CRF | 54.55 ± 1.45 | 59.18 ± 0.73 |
| TRH | 13.55 ± 0.85 | 13.66 ± 0.29 |

naling by these receptors is consistent with the $[Ca^{2+}]_i$ dependence of sGC-derived cGMP production.

Several lines of evidence presented here, however, indicate that $Ca^{2+}$ is not an exclusive signaling pathway for stimulation of cGMP production by GPCRs. GHRH and CRF were found to stimulate sGC in cells bathed in $Ca^{2+}$-deficient medium, a treatment that abolished agonist-induced $Ca^{2+}$ influx. The $Ca^{2+}$-mobilizing action of TRH, but not the agonist-induced cGMP accumulation, was abolished in cells with intracellular $Ca^{2+}$ pools depleted by thapsigargin. In contrast to $[Ca^{2+}]_i$ signals, cGMP production was found to correlate well with cAMP levels, suggesting that GPCRs stimulate the NOS/sGC signaling pathway through the cAMP-dependent mechanism. A decrease in cGMP production observed in cells stimulated with ET-1 and apomorphine also paralleled a decrease in cAMP production. The dependence of cGMP accumulation on cAMP levels was further documented in experiments with CTX, a treatment that elevated the levels of both nucleotides. To directly activate AC, we treated cells with forskolin. Furthermore, to exclude AC-independent actions of forskolin, cells were treated with 8-Br-cAMP, a permeable cAMP analog. In both experiments, a significant increase in cGMP production was observed confirming a hypothesis that cAMP mediates the action of GHRH, CRF, and TRH on cGMP production. Finally, receptor-, CTX-, and forskolin-induced stimulation of cGMP production, but not cAMP production, was reduced in the presence of H89. These results indicate that GPCRs can modulate sGC activity through the AC signaling pathway.

The dependence of sGC activity on the protein kinase A signaling pathway is consistent with several reports indicating that phosphorylation of eNOS makes this enzyme active in the absence of $[Ca^{2+}]_i$ signaling. The serine/threonine protein kinase Ark/PKB-induced phosphorylation of eNOS is well established (45, 46). One report also suggests that eNOS from endothelial cells is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases (47). Earlier studies have also indicated that protein kinase C and protein kinase A can phosphorylate sGC *in vitro*, leading to the facilitation of enzyme activity (48, 49). In intact PC12 cells, phorbol ester-activated protein kinase C also phosphorylates sGC and increases the enzyme activity (50). At the present time, our experiments cannot distinguish between the possible direct actions of protein kinase A on sGC from that mediated by NOS. However, it is unlikely that phosphorylation of eNOS accounts for GHRH-induced cGMP production in somatotrophs because these cells do not express eNOS, but do express nNOS (18), phosphorylation of which leads to a decrease in NO production (51).

A calcium-independent increase in cGMP levels observed in agonist-stimulated cells is also compatible with findings that this ion plays an important role in the control of PDE1 activity in a calmodulin-dependent manner. Phosphorylation of PDEs by protein kinases A and G also modulates the activity of these enzymes (52–54). Thus, calcium depletion/repletion in culture media in our experiments could influence the rate of cGMP degradation. However, that does not provide a rationale for agonist-induced cGMP accumulation, because it was also observed in cells bathed in medium containing 1 mM IBMX, an inhibitor that blocks PDE1 with an $IC_{50}$ of 4 μM, as well as PDEs 2, 3, 4, 5, 6, 7, 10, and 11, with $IC_{50}$ values of 2–100 μM (52). Agonist-induced cGMP accumulation was also observed in cells bathed in the presence of vinpocetine, a specific PDE1 inhibitor, as well as in the presence of specific inhibitors of cGMP-dependent PDEs, dipyridamole, and zaprinast (52). The ability of GHRH, CRF, and TRH to stimulate cGMP accumulation in cells bathed in medium containing a mixture of three PDE inhibitors in high concentrations further argues against the hypothesis that protein kinase A-dependent phosphorylation of PDEs, specifically PDE1A, accounts for down-regulation of their activities and elevation in cGMP levels.

In conclusion, here we show that GHRH, CRF, and TRH receptors in pituitary cells stimulate sGC activity and that this stimulation also occurs when $Ca^{2+}$ signaling is abolished. The experiments argue against the action of these receptors on PDE activity and support the hypothesis that *de novo* cGMP production accounts for a $Ca^{2+}$-independent increase in cGMP levels. Our results further indicate that the coupling of GHRH and CRF receptors and cross-coupling of the TRH receptor to the $G_s$ signaling pathway represents the main mechanism for stimulation of sGC, whereas the coupling of receptors to the $G_{i/o}$ signaling pathway leads to inhibition of cGMP production. The results also indicate that $G_s$-mediated activation of AC and subsequently protein kinase A is required for stimulation of sGC activity. Thus, in addition to $[Ca^{2+}]_i$, up- and down-regulation of AC activity by GPCRs provides an effective mechanism for control of cGMP production.

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